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SHORT COMMUNICATION

Application of a Real-Time PCR Method for *Salmonella* spp., *Escherichia coli*, *Staphylococcus aureus* and *Clostridium perfringens* Detection in Water Samples

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Abstract

The diagnostic assessment of water sanitary state is based mainly on the cultivation of bacteria retained on membrane filters. However classical microbiology methods have a lot of disadvantages. More and more frequently, rapid detection and identification of pathogens present in water is based on molecular biology techniques. The aim of this study was to determine the effectiveness and usefulness of a real-time PCR method, when compared to the recommended bacteria culture method, in diagnostics of pathogens in water samples. The research concerned the detection and identification of main sanitary indicators of water such as: *Salmonella* spp., *Escherichia coli*, *Staphylococcus aureus* and *Clostridium perfringens*. The analyses were conducted in water samples contaminated with the reference material (the aforementioned bacteria) and real environmental samples, which were examined for the presence of nucleic acid of: *Salmonella* spp., *E. coli*, *S. aureus* and *C. perfringens* using a real-time PCR method.

Key words: *C. perfringens*, *E. coli*, *Salmonella* spp., *S. aureus*, real-time PCR, water

Water is not considered to be the habitat of pathogenic bacteria, such as: *Escherichia coli*, *Salmonella species*, *Clostridium perfringens*. Usually, the microorganisms are transferred there directly from human or animal bodies. Sewage can also be a direct source of pollution. Contact with contaminated water may be hazardous for human health. These bacteria are basic indicators of sanitary contamination of surface water, as well as bathing and drinking water.

E. coli and *Salmonella* spp. are Gram-negative bacteria of the family *Enterobacteriaceae*. The first one, *E. coli*, partly forms physiological flora of human and animal colon, but in specific conditions it may become pathogenic (*e.g.* when bacteria migrate to systems other than the intestine or when they produce toxins). Infections in children and adults manifest themselves by watery diarrhea. Less common symptoms of diarrhea are fever, nausea, abdominal pain, stool with blood and mucus (Cennimo *et al.*, 2007). The virulence of *E. coli* strains depends on the adhesion to the gastrointestinal epithelium, and the production of toxins (Chattaway *et al.*, 2011, Kaur *et al.*, 2010). *Salmonella* is the main cause of food poisoning, typhoid fever and paratyphoid fever. Mostly, it is transmitted to humans by contaminated food and water (Ohl and Miller, 2001).

C. perfringens is a Gram-positive, anaerobic, rod-shaped bacterium producing endospores. The gastrointestinal tract of animals and humans is its habitat. *C. perfringens* strains were classified into five types based on the ability to produce four toxins: α, β, ε, ι (Cavalcanti *et al.*, 2004; Kądzielska *et al.*, 2012).

S. aureus is a Gram-positive bacterium of *Staphylococcaceae* family which is found in the upper respiratory tract, on the human skin and the urogenital system. In specific conditions, *S. aureus* may cause diseases such as: acute skin infections, subcutaneous tissues and soft tissues infections, systemic infection, and infection or poisoning associated with the production of toxins. The symptoms of such poisoning are diarrhea, nausea, low blood pressure, septic shock, and even death. The presence of this bacterium does not always entail infection. A large number of humans turn out to be asymptomatic carriers of the bacterium (Bien *et al.*, 2011).

The diagnostic assessment of water sanitary state is based mainly on the cultivation of bacteria retained on membrane filters. The major disadvantage of this

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cultivation method is long waiting time for the results. Moreover, it is often limited by composition of a medium used to grow a particular culture. Some pathogens may grow faster on a particular medium, which inhibits the growth of others (Toze, 1999). Medium ingredients provide microorganisms with conditions approximately similar to natural ones (Lee *et al.*, 2006). However, the methods of classical microbiology are inexpensive and uncomplicated (Rogers *et al.*, 2011). Currently, molecular biology techniques based on polymerase chain reaction (PCR) are used in microbiological diagnosis. They enable rapid detection and identification of all pathogens present in water (Valasek and Repa, 2005; Wong and Medrano, 2005). The most widespread molecular method of diagnosis, real-time PCR (quantitative PCR, qPCR), is characterized by high sensitivity, specificity, efficiency and quickness (Wong and Medrano, 2005; Yang *et al.*, 2002; Smith and Osborn, 2008). The application of a real-time PCR method enables to identify genetic material of microorganisms directly from environmental samples (Kacprzak *et al.*, 2012; Toze, 1999). Unfortunately, the detected genetic material can be derived from both dead and living cells (Fey *et al.*, 2004). The risk of contamination in a real-time PCR method is very small, whereas the capacity of this method is very high (Dixit and Shaker, 2009; Heid *et al.*, 2012). The unquestionable advantage of a real-time PCR method is the ability to detect living bacteria that are dormant. These bacteria are not capable of growing on microbiological media (VBNC, viable but nonculturable) (Rogers *et al.*, 2011; Toze, 1999; Dufour and Stelma, 2010).

The aim of this study was to determine the effectiveness and usefulness of a real-time PCR method for identification of pathogenic bacteria which are found in water samples: *Salmonella* spp., *E. coli*, *C. perfringens* and *S. aureus*.

In the first stage of the experiment, the detection limit of tested pathogens was determined. Reference materials EasiTab™ (BioSenate, Bury, UK) containing lyophilized microorganisms: *E. coli* – NCTC 9001, *C. perfringens* – NCTC 8797, *S. aureus* – NCTC 6571 and reference material MicroPellet (Mecconti, Mondorf-les-Bains, Luxembourg) containing a lyophilized strain of *S. enterica* – ATCC 14028 were used to produce stock solutions of bacteria. Each solution had a volume of 100 ml and contained the following number of microorganisms: *E. coli* – 2.1×10^4 colony forming unit (CFU), *C. perfringens* – 6.9×10^4 CFU, *S. aureus* – 3.0×10^4 CFU, *S. enterica* – 6.2×10^4 CFU. Then, the dilution series of contaminated samples at concentrations ranging from 10^0 to 10^{-4} were prepared. Sterile water was used for dilution. Each dilution contained a known number of microorganisms. This helped to define the limits of detection in a real-time PCR method. Extraction of DNA and pathogen detections in individual dilutions were conducted using methods and reagents described below.

Then, contaminated samples were prepared using reference materials: EasiTab™ and MicroPellet. Lyophilized bacteria was dissolved in sterile water according to the manufacturer's instructions. There were fifteen contaminated samples. Each solution had a volume of 1500 ml. In addition, twenty-five blank samples (sterile water) were prepared (Table I). All samples were duplicated. One part was analyzed using a real-time PCR method, and the other using cultivation methods. Moreover, fifty environmental samples of water were obtained for analysis. Environmental samples were collected from surface sources of water (streams, rivers, lakes), swimming pools, deep water intakes, surface water intakes and mixed water intakes. Each sample had a volume of 1500 ml and was duplicated. One part was analyzed using a real-time PCR method and the other using cultivation methods.

At the beginning of a water sample analysis, DNA extraction from bacteria was performed with a commercially available kit Aqua Screen® FastExtract (Minerva Biolabs, Berlin, Germany). The whole volume of water samples was filtered through membrane with a pore size of 0.45 μm, which was attached to the kit. This step was identical to the steps of water samples preparation for cultivation methods. The next stages were prepared according to the manufacturer's instructions, however cell lysis was modified. The whole volume of lysate was used for incubation at 56°C, which resulted in sensitivity increase and increased recovery of the genetic material from the filter.

The detection of pathogens in contaminated and environmental samples was performed using LightCycler 480 II (Roche, Basel, Switzerland). The amplification reactions were performed using commercially available kits: SureFood BAC *Staphylococcus aureus* PLUS LC (Congen Biotechnology, Berlin, Germany), SureFood BAC *Clostridium perfringens* PLUS LC (Congen Biotechnology, Berlin, Germany), Foodproof *Salmonella* Detection Kit (Biotecon Diagnostics, Potsdam, Germany), Foodproof *E. coli* and *Shigella* Detection Kit (Biotecon Diagnostics, Potsdam, Germany). The thermal profile of *Salmonella* spp. and *E. coli* analysis for a real-time PCR was as follows: pre-incubation at 37°C for 2 min, initial denaturation at 95°C for 10 min, and then 45 cycles consisting of the following temperatures and time intervals: 95°C for 5 s, 59°C for 35 s and 72°C for 15 s. The last step was cooling at 40°C for 30 s. Whereas, the thermal profile of *S. aureus* and *C. perfringens* analysis was as follows: denaturation at 95°C for 5 min, and then 45 cycles consisting of the following

Table I
The number of contaminated samples studied by a real-time PCR method and the cultivation methods.

Sample type	*Salmonella* spp.		*E. coli*		*C. perfringens*		*S. aureus*	
	Contaminated (positive) samples	Blank samples	Contaminated (positive) samples	Blank samples	Contaminated (positive) samples	Blank samples	Contaminated (positive) samples	Blank samples
Sample number	3	7	3	7	4	6	5	5

Table II
The crossing point (Cp) values for analyses by a real-time PCR method.

Contaminated samples	A number of samples	Crossing points (Cp)		Mean	Standard deviation	Relative standard deviation	Variance
Salmonella spp.	3	32.83	33.02	32.92	0.134	0.0041	1.67×10^{-5}
		30.74	30.77	30.75	0.021	0.0007	4.76×10^{-7}
		32.11	32.20	32.15	0.064	0.0020	3.92×10^{-6}
E. coli	3	30.73	30.63	30.68	0.071	0.0023	5.31×10^{-6}
		30.79	30.79	30.79	0	0	0
		28.70	28.68	28.69	0.014	0.0005	2.43×10^{-7}
C. perfringens	4	37.77	37.49	37.63	0.198	0.0053	2.77×10^{-5}
		36.64	38.16	37.40	1.075	0.0287	8.26×10^{-4}
		37.48	35.90	36.69	1.117	0.0304	9.27×10^{-4}
		37.04	35.64	36.34	0.990	0.0272	7.42×10^{-4}
S. aureus	5	35.04	35.23	35.13	0.134	0.0038	1.46×10^{-5}
		35.21	34.76	34.98	0.318	0.0091	8.27×10^{-5}
		37.56	37.39	37.47	0.120	0.0032	1.03×10^{-5}
		37.05	37.60	37.32	0.389	0.0104	1.09×10^{-4}
		37.43	38.87	38.15	1.018	0.0267	7.12×10^{-4}

temperatures and time intervals: 95°C for 15 s, 60°C for 15 s. All assays were conducted in duplicate.

The crossing point (Cp) values were determined automatically using the Second Derivative Maximum method. The results interpretation in *Salmonella* spp. and *E. coli* analyses was conducted in the Red 640 channel (excitation wavelength/emission wavelength: 498–640 nm) while internal control was analyzed using the Cy 5/Cy 5.5 channel (excitation wavelength/emission wavelength: 498–660 nm). The FAM channel (excitation wavelength/emission wavelength: 465–510 nm) was used for *S. aureus* and *C. perfringens* analyses while internal control was analyzed using ROX/Texas Red channel (excitation wavelength/emission wavelength: 533–610 nm).

The study of water using reference methods of classical microbiology was performed to demonstrate the growth on selective media. Microbiological studies were conducted according to the test procedures based on the standards and regulations of the National Institute of Public Health – National Institute of Hygiene in Warsaw.

All contaminated samples were analyzed by a real-time PCR method for the presence of *Salmonella* spp., *E. coli*, *S. aureus* and *C. perfringens*. The increase of fluorescence was observed in contaminated samples, whereas no increase of fluorescence was observed in blank samples (data not showed). The positive results of tested pathogens detection were characterized by a logarithmic increase of fluorescence. The Table II shows Cp values for particular analyses. The variation coefficient of Cp values was 1.52%. The assessment of reaction parameters shows that the amplification was conducted correctly.

The results obtained by a real-time PCR method were confirmed by the cultivation method. In this study, fifteen contaminated positive samples and twenty-five negative samples were obtained using a molecular method and a classical method of microbiology (Table III). The detection limits were determined for each pathogen using a dilution series of known concentrations. The detection limit was respectively: *Salmonella* spp. – 6.2×10^{2} CFU, *E. coli* – 2.1×10^{3} CFU, *S. aureus* – 3.0×10^{2} CFU, *C. perfringens* – 6.9×10^{1} CFU. Apart from detection limit, other validation parameters of real-time PCR method were collected in the Table IV. The additional analysis of validation parameters confirmed studies and results reliability.

Table III
A real-time PCR method and the cultivation methods for detecting *Salmonella* spp., *E. coli, S. aureus, C. perfringens.*

Object of study	Positive samples number		Negative samples number		Percentage of certified results (%)
	Real-time PCR	Culture method	Real-time PCR	Culture method	
Contaminated samples of water	15	15	25	25	100
Environmental samples of water	3	0	47	50	94

Table IV
Basic parameters of the validation of real time PCR method.

	Salmonella spp.	*E. coli*	*S. aureus*	*C. perfringens*
Results correctness [%]	AC* = 100 SE* = 100 SP*=100	AC* = 100 SE* = 100 SP* = 100	AC* = 100 SE* = 100 SP* = 100	AC* = 100 SE* = 100 SP* = 100
Detection limit [CFU]	6.2×10^3	2.1×10^3	3.0×10^3	6.9×10^3
Repeatability [%]	100	100	100	100
Reproducibility [%]	100	100	100	100
Method uncertainty [%]	1.8	1.8	1.9	3.0
Relative accuracy [%]	100	100	100	100
Relative sensitivity [%]	100	100	100	100
Relative specificity [%]	100	100	100	100

* AC – accuracy, SE – sensitivity, SP – specificity

The examination of fifty environmental water samples by reference cultivation methods and an alternative molecular biology method revealed differences. In three samples which were negative in the cultivation method, the presence of *S. aureus* genetic material was confirmed (Table III).

Urbanization and industrialization result in water pollution in the developed and developing countries. Water can be the source of pathogenic bacteria which are responsible for spreading infections in the whole world (Dixit and Shaker, 2009). To avoid this, you need to improve diagnosis of these organisms. Many published reports describe using real-time PCR techniques to facilitate the identification of pathogens detected in various environmental samples (Ibekwe and Grieve, 2003; Ahmed *et al.*, 2008; Lee *et al.*, 2006).

The results obtained in this study confirmed the importance of a real-time PCR method for a fast diagnosis of pathogens which are present in water. For all tested pathogens such as: *Salmonella* spp., *E. coli*, *S. aureus* and *C. perfringens* effectiveness of the method was confirmed. The results obtained by a real-time PCR assay were confirmed by a cultivation method. This is important because it provides a high quality of diagnosis and accuracy of analyses. The differences between the results of testing environmental samples resulted from restrictions of a cultivation method, which was due to a random choice of representative bacterial colonies.

Application of a real-time PCR assay allowed to reduce significantly the waiting time for results. When every minute counts, it is very important to confirm or rule out the possibility of bacterial infection. The isolation of genetic material without a pre-multiplication reduces the identification time of pathogens which are present in water. Aqudelo *et al.* (2010) draw similar conclusions on the cultivation method and a real-time PCR assay in *E. coli* and fecal enterococci diagnosis from water samples. Rintilla *et al.* (2011) analyzed stool samples using the same method.

Molecular biology techniques have many opponents, and cultivation methods are the "golden mean" in the water diagnosis. In recent years, a lot of molecular techniques have been approved for the detection of pathogens, but unfortunately there are still difficulties such as: lack of standardization, reproducibility and stability of the isolated genetic material (Beneduce *et al.*, 2007, Noble and Weisberg, 2005). However, it can be of great significance to quickly obtain the results of pathogens detection in water. This allows the sanitary services to respond immediately and suppress the public health problem almost at the beginning.

Summarizing, this paper confirmed the efficacy of a real-time PCR method in the diagnosis of the presence of pathogenic bacteria in water. Moreover, a real-time PCR can be an important tool for fast and specific microbiological diagnosis.

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